Bronchopulmonary Cellular Response to Aluminum and Zirconium Salts

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The bronchopulmonary cellular immunological response to repeated intratracheal inoculation of aluminum chlorhydrate, sodium zirconium lactate, and zirconium aluminum glycine was examined in rabbits. Results of a dose-response experiment using 0.1, 1.0, and 10.0-mg intratracheal inoculations of each metallic salt demonstrated significant bronchopulmonary histopathology in the 10.0-mg dose-response groups only. Acute lesions were histologically characterized by an inflammatory response centered around respiratory bronchioles. Although epithelioid cell formation was evident in 10.0 mg of aluminum salt (aluminum chlorhydrate and zirconium aluminum glycine) -injected animals, no well-defined granulomas characterized by an orderly arrangement of epithelioid cells, lymphocytes, and giant cells were evident in any of the experimental groups employed. All three metallic salts induced "activated" bronchopulmonary macrophages as determined by an in vitro phagocytic assay. This activation was likely nonimmunological since no measurable differences were observed in metallic salt-induced delayed skin reactivity or migration inhibition factor production between inoculated and uninoculated rabbits. The above observations suggest that aluminum and zirconium salts administered in comparatively high dosage via the respiratory tract route can induce respiratory bronchiolitis and activation of alveolar macrophages in the absence of demonstrable delayed hypersensitivity.

The association of the element zirconium with dermal granuloma formation was first cited by Weber et al. in 1956 (15). Characterized by a chronic, papular eruption of the axillae after application of zirconium-containing deodorant sticks, zirconium-related dermal granuloma formation has since been categorized as a probable "allergic" granuloma. In an extensive analysis of the subject, Shelley and Hurley confirmed positive skin reactivity to zirconium in 2 of 50 human male subjects after intensive, prolonged, daily application of a commercial zirconium deodorant stick (13). Histologically, "allergic" zirconium-related granulomas have been distinguished from nonallergic granulomas by both the presence of epithelioid cells as the predominant component of the cellular infiltrate and the organization of these cells into nests or tubercles (4, 13).

In view of the dermal-sensitizing potential of zirconium and the reported immunological pathogenesis of zirconium-related dermal granuloma formation, the use of zirconium in aerosol form has been questioned. It is the purpose of this study to examine and compare the bronchopulmonary (BP)-sensitizing potential of two zirconium-containing salts, sodium zirconium lactate (NZL) and zirconium aluminum glycine (ZAG), with that of aluminum chlorhydrate (ACH), an antiperspirant which thus far has not been implicated as a sensitizing agent in humans.

MATERIALS AND METHODS

Animals. Outbred, female New Zealand white rabbits, weighing 2 to 3 kg, were obtained locally and housed in stainless-steel cages. The animals were allowed food pellets and water ad libitum.

Metallic salts and bacteria. ACH, NZL, and ZAG were supplied in powder form by the Procter and Gamble Co., Inc., Cincinnati, Ohio. A clinical isolate of *Listeria monocytogenes* was maintained on 5% blood agar and grown overnight in tryptic soy broth (GIBCO) for use in the macrophage phagocytic assay.

Intratracheal inoculations and intradermal skin testing. Animals were lightly anesthetized with pentobarbital administered via a peripheral ear vein. The neck was shaved, hyperextended, and swabbed with 95% ethanol, followed by direct intratracheal (i.t.) inoculation with a 22-gauge needle. Rabbits received three i.t. injections of either 0.1, 1.0, or 10.0 mg of each metallic salt suspended in 3 ml of sterile saline administered at 3-day intervals. All metallic salt solutions were freshly prepared before each inoculation. Intradermal skin tests were performed 3 days before sacri-

fice excluding those animals sacrificed 1 day after the last i.t. inoculation. Skin test sites were shaved and depilated, and 0.1 ml of metallic salt in sterile saline was injected intradermally. The diameter of erythema and/or induration was measured at 24, 48, and 72 h after skin testing. The skin test dose for each metallic salt ranged from 25 to 100 μg.

BP histological examination. Four rabbits were sacrificed for each of the three concentrations of metallic salts at 1, 14, and 28 days after the last i.t. inoculation. Animals were sacrificed by air embolism, and the lungs were excised according to the method of Myrvik et al. (11). After inflation and fixation under constant pressure (20 cm of water) with a phosphate-buffered 10% Formalin–2% gluteraldehyde solution, each lobe was sectioned, stained with hematoxylin and eosin, and examined by light microscopy.

Alveolar macrophage cultures and migration inhibition factor assay. Rabbit BP cells were harvested according to the method of Myrvik et al. (11), using Hanks balanced salt solution (HBSS) (Microbiological Associates, Bethesda, Md.) as the supporting medium. When erythrocytes were present in the cell population, they were lysed with 0.8% tris-(hydroxymethyl)aminomethane-buffered ammonium chloride and the cells were washed with HBSS. BP cells were adjusted to 2.5×10^6 to 5.0×10^6 cells per ml in RPMI-1640 medium (GIBCO). A 100-ml medium contained 1,000 U of penicillin, 1,000 μg of streptomycin, 2,500 μg of kanamycin, 0.5 ml of 200 mM glutamine, and 15% heat-inactivated autologous rabbit serum drawn before i.t. inoculation. Two milliliters of the cell suspension was pipetted into 35-mm-diameter plastic tissue culture dishes (Falcon Plastics, Oxnard, Calif.) and incubated at 37°C in 95% air–5% CO_2 (14). A direct migration inhibition factor assay of alveolar wash cells was performed according to the method of David and David (2). The cell suspensions were drawn into capillary tubes; tubes were sealed by flaming, and cells were packed by centrifugation (50 × g for 6 min). Tubes were cut just below the cell interface and placed in Sykes-Moore chambers containing RPMI-1640 with 15% normal autologous serum, antibiotics, and the appropriate metallic salt (100 μg/ml). After 24 h of incubation at 37°C, the area of migration patterns was projected and drawn. Migration areas were measured by planimetry, and inhibition greater than 20% was considered positive.

Phagocytic assay and quantitation of BP macrophage function. A direct phagocytic assay using viable *L. monocytogenes* as the test organism was performed essentially as described by Johnson et al. (8). BP wash cells were incubated overnight at 37°C in 95% air–5% CO_2 as described above. After removal of nonadherent cells by washing with HBSS, the adherent cell population was challenged with varying multiplicities of infection (130 to 300) of *L. monocytogenes* suspended in RPMI-1640 (without antibiotics) supplemented with 15% heat-inactivated autologous rabbit serum. After incubation for 30 min at 37°C in 95% air–5% CO_2, the culture dishes were washed six times with HBSS to remove nonphagocytized bacteria. The macrophage monolayers were then lysed by adding distilled water for 30 min and serially diluted and plated on blood agar for bacterial enumeration. Triplicate samples were processed for each macrophage

preparation. Bacterial colonies were counted after incubation on blood agar at 37°C for 48 h.

For quantitation of BP macrophage phagocytic activity, the number of macrophages adherent to culture dishes after overnight incubation were counted in a hemacytometer after washing the monolayers with HBSS and removing adherent cells with a Teflon scraper. A phagocytic index for each assay was calculated by determining the number of viable bacteria per adherent cell after 30 min of incubation with *L. monocytogenes*.

Statistical analyses. Calculation of arithmetic means, standard error of means, and variance were performed with a computer program. Statistical comparisons of alveolar wash cell numbers were obtained by the Student's *t*-test, whereas macrophage phagocytic activities were compared by linear-regression analysis.

RESULTS

BP histopathology. To appraise the BP cellular-sensitizing potential of both aluminum and zirconium salts, a dose-response experiment was initially conducted. Since each metallic salt when dissolved in sterile saline produced a pH ranging from 4.5 to 5.0, 12 rabbits were inoculated with pH 4.5 saline to control for the effect on the lung of an acid pH. BP histological appraisal at 1, 14, and 28 days postinoculation revealed normal lung histology in all saline-injected animals and those inoculated with 0.1 and 1.0 mg of metallic salt.

The pulmonary reaction to i.t. inoculation with 10 mg of ZAG was more severe than that produced with 10 mg of ACH. Inoculation with 10 mg of NZL produced the least severe lung changes of all three metallic salts tested. BP histopathology in the 10 mg of ZAG-injected groups consisted of an initial (1 day after the last i.t. injection) widespread mild-to-moderately severe respiratory bronchiolitis (Fig. 1). Interstitial tissue was edematous and infiltrated with a mixture of polymorphonuclear leukocytes and lymphocytes. Many alveoli and respiratory bronchioles were filled with alveolar macrophages and polymorphonuclear leukocytes, some of which had pyknotic nuclei. There were several areas of frank suppuration, and all abnormalities were maximal around respiratory bronchioles. At 14 days after the last injection most sections were normal, with the remainder demonstrating scattered areas of interstitial thickening as a result of edema and infiltration with lymphocytes and epithelioid cells, the latter characterized by vesicular nuclei, eosinophilic granular cytoplasm and indistinct cytoplasmic borders (Fig. 2). By 28 days, all animals exhibited no residual histological abnormalities.

The acute (1 day after the last injection) response to 10 mg of ACH was predominantly interstitial and characterized by edema and lym-
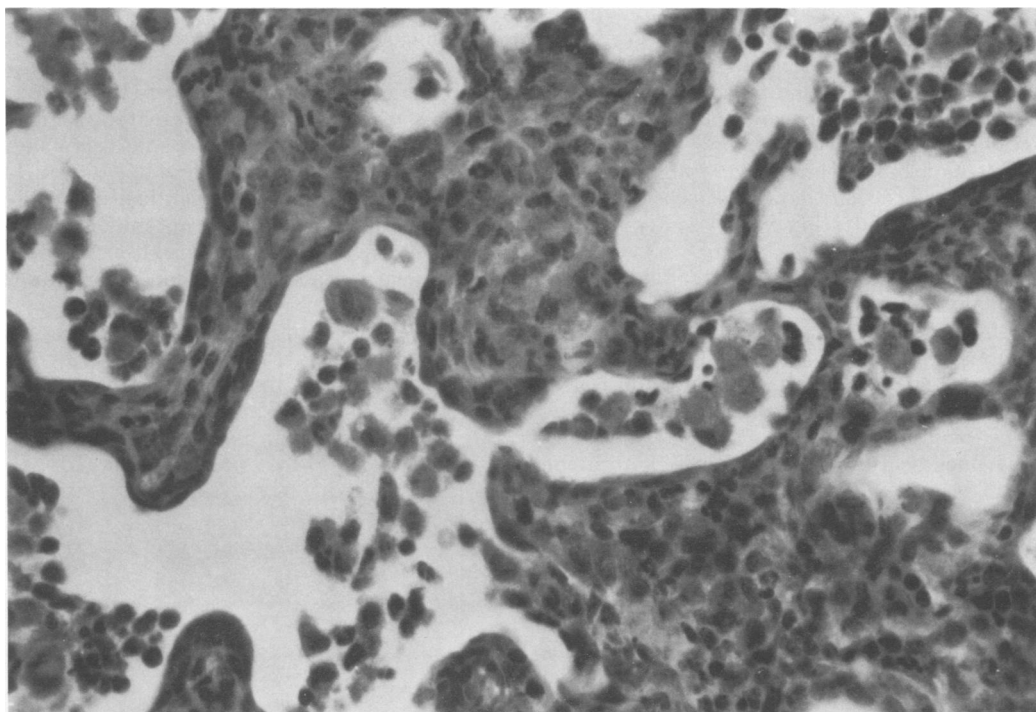
FIG. 1. *Pulmonary histology 1 day after the last of three 10-mg i.t. inoculations with ZAG. Depicts alveolar wall thickening with mononuclear and polymorphonuclear cells in the interstitium and alveolar spaces. Hematoxylin and eosin. ×450.*
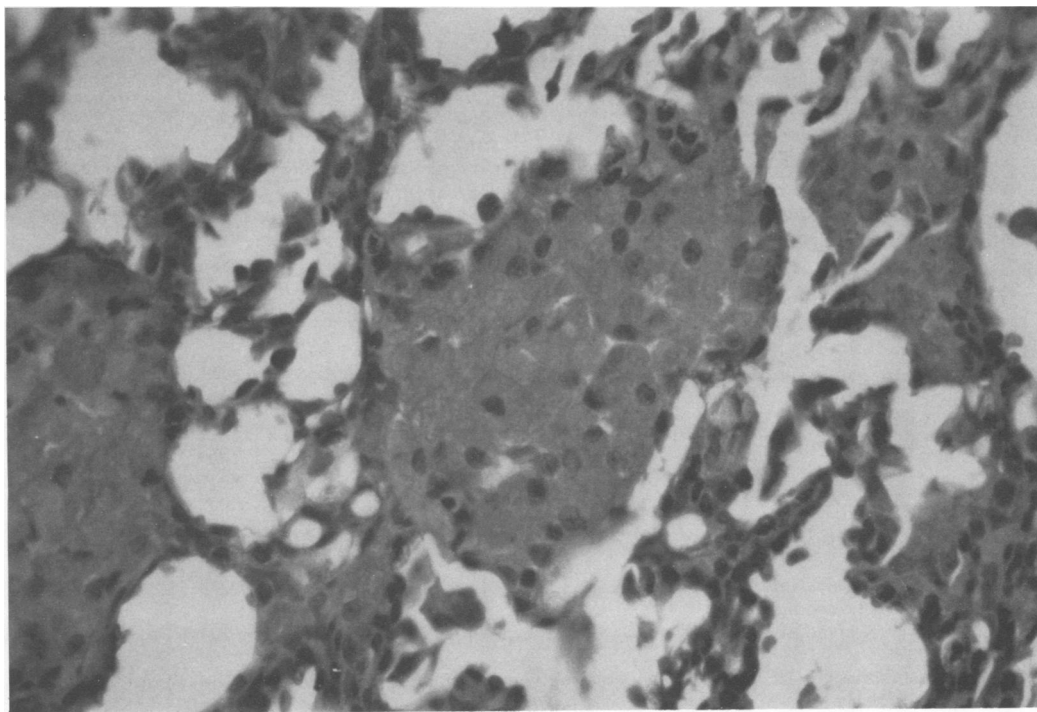

FIG. 2. *Pulmonary histology 14 days after the last of three 10-mg i.t. inoculations with ZAG. Shows epithelioid cell collections both in the interstitium and alveoli. Hematoxylin and eosin. ×450.*

phatic distension with lymphocytes and epithelioid cells. These abnormalities were centered around respiratory bronchioles. At 14 days, there was persistance of interstitial disease with the appearance of focal alveolar collections of macrophages. Twenty-eight days after the last i.t. injection most sections were normal in appearance and contained only an occasional focus of interstitial infiltration with lymphocytes and epithelioid cells.

The acute lesions in NZL-treated rabbits were minimal and consisted of few areas of interstitial lymphocyte infiltrates. At 14 and 28 days, the pulmonary histology was normal.

Mononuclear cell migration into lungs after metallic salt inoculation, as first suggested by the histological alterations of alveolar wall thickening and focal collections of cells within the alveoli, was further supported by direct quantitation of BP wash cell numbers. Although a limited number of animals were examined in all groups, a statistically significant increase in alveolar wash cell number was observed after inoculation of all three metallic salts (Table 1). Morphological differentiation of BP wash cells confirmed an average mononuclear contribution ranging from 85 (1 day after the last i.t. injection) to 95% (14 and 28 days after the last i.t. inoculation).

BP cellular functional assessment. With an in vitro phagocytic assay, the function of BP macrophages obtained from 10.0 mg of metallic salt-inoculated rabbits was examined at 1, 14, and 28 days after the last i.t. inoculation. By expressing macrophage activation as increased phagocytosis of viable *L. monocytogenes*, alveolar wash cells obtained from aluminum and zirconium metallic salt-inoculated rabbits were activated when compared with BP wash cells obtained from uninoculated or saline-injected controls (Table 1).

A phagocytic comparison between metallic salt-exposed activated BP wash cells (Table 1) and alveolar macrophages obtained from control animals (saline injected) is presented in Fig. 3. It is stressed that phagocytic responses by individual cell preparations in all groups (day 1, 14, and 28 postexposure) were identical within the group (i.e., enhancement or no enhancement). For clarity, therefore, metallic salt-exposed BP macrophage groups with responses similar to controls are not shown. By increasing the multiplicity of infection, both control and metallic salt-exposed BP macrophages demonstrated an increase in bacterial phagocytosis. However, despite variations in the multiplicity of infection, statistically significant increases greater than
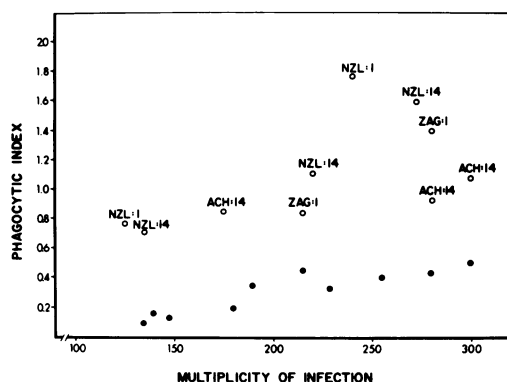

FIG. 3. *Phagocytosis of viable L. monocytogenes by BP macrophages obtained from uninoculated or saline-inoculated controls (●) and metallic salt-inoculated rabbits (○). Multiplicity of infection represents the number of viable bacteria added per adherent macrophage. Phagocytic index equals the number of viable bacteria engulfed per adherent macrophage after 30 min of incubation.*

TABLE 1. *BP cellular response to 10-mg i.t. inoculations of ACH, NZL, and ZAG*

Days post-inoculation[a]	Cellular response					
	No. of alveolar wash cells with:[b]			Macrophage function with:[c]		
	ACH	NZL	ZAG	ACH	NZL	ZAG
1	14.8 ± 0.70^{d} (2)[e]	15.7 ± 0.70^{d} (2)	25.4 ± 1.40^{d} (2)	No activation	Activation	Activation
14	26.1 ± 1.39^{d} (3)	16.4 ± 0.26^{d} (3)	17.5 ± 1.79^{d} (3)	Activation	Activation	No activation
28	14.5 ± 1.30^{d} (2)	10.5 ± 1.19 (4)	17.2 ± 1.10^{d} (2)	No activation	No activation	No activation

[a] Days after last i.t. inoculation.
[b] Mean number of alveolar wash cells $\times 10^{7} \pm$ standard error (uninoculated and/or saline-injected controls equals 9.2 ± 0.36).
[c] Activation, enhanced macrophage phagocytosis of viable *L. monocytogenes*.
[d] Differs significantly from normal value at $P < 0.001$.
[e] Number of animals per group.

twofold were evident between metallic salt-exposed activated and control alveolar wash cells ($P < 0.01$).

Skin test and migration inhibition of alveolar macrophages. To correlate the observed BP macrophage activation with established correlates of cellular hypersensitivity, metallic salt-delayed skin reactivity and inhibition in migration of alveolar wash cells were measured. Intradermal skin tests were performed on all inoculated animals excluding those sacrificed 1 day after the last i.t. inoculation. An i.t. exposure to all three metallic salts produced no measureable differences in erythema or induration between inoculated or uninoculated rabbits after intradermal injection of the appropriate chemical. Direct migration in inhibition factor assay of four separate BP wash cell preparations in each of the four inoculated groups which demonstrated phagocytic activation (Fig. 3) revealed no inhibition in cellular migration in the presence of metallic salt. These observations argue against the induction of both local and systemic delayed hypersensitivity to metallic salt inoculation.

DISCUSSION

Results of this study indicate that i.t. inoculation of rabbits with aluminum and zirconium salts leads to the development of a dose-related acute inflammatory respiratory bronchiolitis but produces no well-defined pulmonary granulomas. Both metallic salts also induced activated BP macrophages, as determined by an in vitro phagocytic assay, in the absence of demonstrable delayed hypersensitivity. Taken together, these observations suggest a lack of BP sensitizing and granulomagenic potentials for aluminum and zirconium.

The pulmonary lesions observed after i.t. administration of ACH, NZL, and ZAG were similar to those previously reported to follow aerosol exposure (1, 3). Although a rank order of ZAG > ACH > NZL could be established for the ability to induce pulmonary lesions, lung sections obtained from 14- and 28-day postinoculation rabbits demonstrated a gradual resolution to normal pulmonary architecture. Indeed, it should be stressed that significant pulmonary lesions were observed only after multiple 10.0-mg i.t. metallic salt inoculations.

The induction of epithelioid cell formation by ACH and ZAG raises the possibility that aluminum might play a role in epithelioid cell granuloma production conceivably through conjugation to animal proteins and creation of an immunogenic stimulus. In this respect, several histological characteristics are generally employed to distinguish allergic granulomas. Of primary differentiation is the arrangement or organization of epithelioid cells into nests or tubercles which are thought to be a common feature of the allergic (high turnover or hypersensitivity) granuloma (4). In addition to histological characterization, allergic granulomas develop in only a small number of the total exposed population and can be elicited following exposure to trace amounts of injected material (13). In the present study, the formation of epithelioid cells by aluminum salts likely represented development of "foreign body-low turnover" granulomas of a nonimmunogenic nature, since these cells did not appear as well-defined granulomas and they were noted in all animals in a dose-dependent manner. In view of the predominantly mononuclear cell influx into the lung after i.t. metallic salt inoculation, and the established link between immunological sensitization, lymphokine production, and resultant macrophage activation (5, 9, 12), our finding of increased BP macrophage phagocytic activity raises the possibility that this activation was mediated by immunological events. This conclusion seems unlikely, however, since macrophage activation was not accompanied by positive delayed skin reactivity to ACH, NZL, or ZAG and there was no evidence for migration inhibition factor production by alveolar wash cells following i.t. metallic salt exposure. Furthermore, recent analysis of normal BP macrophages exposed to these metallic salts in vitro has revealed increased production and secretion of lysosomal hydrolases (K. Kang and J. E. Salvaggio, submitted for publication). The current, overall findings, therefore, would support a direct interaction between metallic salt and macrophage, devoid of immunological intervention.

The element zirconium and its metallic salts have long been recognized as safe and essentially harmless compounds for both humans and animal (6, 7, 10). The BP histological and cellular functional assessment which follows exposure via the respiratory tract route supports this characterization by suggesting a lack of sensitization to both aluminum and zirconium salts.

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